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erbB-2/neu amplification and overexpression is the most common genetic alteration in breast cancer. As a growth factor receptor and member of the EGFR family, erbB-2 signaling is dependent upon target tissue expression of erbB-2 regulating growth factors and other EGFR family members. To determine the role erbB-2 plays in malignant mammary gland development I examined expression of each EGFR family member and their ligands during normal development of the mouse mammary gland. I found that each receptor is expressed throughout mammary gland development. In contrast, mammary gland expression of the erbB-2 agonists AR and HRG is developmentally regulated - AR being expressed in virgin mice and HRG expressed during pregnancy. This result suggested that erbB-2 agonists may drive mammary gland development. To test this hypothesis I implanted pellets containing HRG within mammary glands of virgin mice. I found that HRG could induce differentiation of mammary epithelium in this system. I predict that erbB-2 expressing breast tumor cells may be induced to differentiate by amplifying the HRG signaling pathway in these cells. I am currently designing experiments in an attempt to decipher the HRG signaling pathway.

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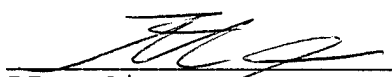

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Introduction

The *neu/erbB-2/HER-2* protooncogene is overexpressed in 15-40% of human breast carcinomas (reviewed in 1). Amplification and overexpression of erbB-2 is associated with poor disease prognosis especially in lymph node positive patients (2, 3, 4). A role for erbB-2 in breast cancer is also supported by the following observations i) overexpression of erbB-2 in cultured fibroblasts is sufficient to induce cellular transformation (5, 6, 7) ii) constitutively active erbB-2 mutants are extremely potent transformation agents (8) and iii) erbB-2 overexpression in the mammary epithelium of transgenic mice results in mammary tumor development (9). Taken together, these results suggest that *erbB-2* amplification is under positive selection in malignant cells and erbB-2 overexpression confers a cellular growth advantage. Therefore, an understanding of the mechanisms which regulate erbB-2 signaling will undoubtedly provide important insights into breast cancer development and progression, and the role(s) erbB-2 plays in these processes.

ErbB-2 is a member of the epidermal growth factor receptor (EGFR) family of receptor tyrosine (tyr) kinases (10). Other members of this receptor family include erbB-3 (11) and erbB-4 (12). Since erbB-2 is a putative growth factor receptor, the role of erbB-2 in the etiology of breast cancer can only be understood in the context of growth factors which regulate erbB-2 signaling.

The EGF-related growth factors are a family of single chain polypeptides characterized by the presence of a consensus sequence (EGF homology domain) containing six cysteine residues of defined spacing. Disulfide bond formation between the cysteine residues results in a three looped secondary structure which distinguishes this family of growth factors (13, 14). EGF family members include EGF, transforming growth factor α (TGFα), amphiregulin (AR), epiregulin, heparin-binding EGF, β-cellulin (βC), and cripto-1 (CR-1). With the exception of CR-1, each EGF family member tested can bind with strong affinity and activate the EGFR (reviewed in: 14). Moreover, in cells which co-express EGFR and erbB-2, EGFR agonists induce the activation of both EGFR and erbB-2.

Significantly, EGFR agonists do not bind to or stimulate erbB-2 signaling when erbB-2 is expressed by itself (15, 16, 17, 18). These apparently contradicting observations can be explained through a proposed mechanism where agonist-driven EGFR activation results in EGFR/erbB-2 heterodimer formation and subsequent receptor cross-phosphorylation (19, 20, 21, 22). This mechanism has been termed "transmodulation" (reviewed in: (1, 23). Several EGF family members are expressed in human breast cancer cell lines (reviewed in: (14)). Moreover a substantial body of evidence suggests that the EGF family of growth factors and their cellular receptors play an important role in both normal and malignant mammary gland development (reviewed in: (1, 14, 24, 25, 26, 27, 28, 29).

A distinct subfamily of the EGF-related growth factors has been identified whose members do not bind the EGFR. This EGF-related group of growth factors were originally identified as polypeptides which bind and activate erbB-2/neu and were thus referred to as the neuregulins (NRGs). The NRGs are a large family of gene splice variants also referred to as neu differentiation factors (30, 31), heregulin α (HRGα) and heregulin β (HRGβ)(32), gp30 (33), acetylcholine receptor inducing activity (34, 35), and glial cell growth factors (36). Although, NRG/HRG were first identified by their ability to induce tyrosine phosphorylation of erbB-2, subsequent observations indicate that NRG/HRG cannot bind erbB-2 in solution or when erbB-2 is expressed on the surface of cells (37, 38, 39, 40, 41). Rather these growth factors bind to and activate two additional members of the EGFR family, erbB-3 (40, 41, 42, 43) and erbB-4 (12, 37, 39, 41). One of these latter receptors must be present to regulate erbB-2 activation by NRG/HRG, presumably through erbB-2/erbB-3 or erbB-2/erbB-4 heterodimer formation (39, 40, 42, 43).

Most recently, a group of EGF-like growth factors structurally related to HRG have been identified and referred to as neuregulin-2 (NRG2)(44, 45). As with HRG (now termed NRG1), NRG2 also directly activates erbB-3 and erbB-4 and can activate erbB-2 but only when erbB-2 is co-expressed with erbB-3 and erbB-4 (44). However, differences in the cellular expression of NRG1 and NRG2 (44, 45) suggests that these two growth factors are functionally distinct.

Normal breast tissue expression of the four EGFR family members EGFR (46, 47), erbB-2 (47, 48), erbB-3 (49), and erbB-4 (49), and several agonists including EGF (50), TGFα (50, 51, 52, 53), AR (54, 55), CR-1 (54, 55), and NRG/HRG (49) has been detected. As described above, cellular signaling via EGFR family members is thought to occur through growth factor driven receptor transmodulation. Furthermore, different growth factors may activate a unique subset of receptors (56, 57). Thus a potentially complex receptor tyrosine kinase signaling repertoire within the developing mammary gland emerges coordinated by the tissue expression of: i) the activating growth factor and ii) receptor transmodulation partners. Moreover, recent evidence indicates that the *in vitro* cellular response of receptor signaling can be radically different depending upon the transmodulation partners and the activating growth factor (56, 57, 58). Based upon these *in vitro* observations one would predict that signaling by receptor tyrosine kinases also induces a diversity of important cellular responses *in vivo*. Indeed, EGFR signaling appears to play an essential role in the epithelial development of several mouse organs and tissues (13, 59, 60, 61, 62, 63). ErbB-2 is also expressed in a variety of embryonic and adult tissues (reviewed in: 1) and has multiple essential functions in mouse neural and cardiac development (64). Interestingly, mice lacking the erbB-4 receptor have cardiac development defects similar to those observed in erbB-2 null mice (64, 65). These results suggest that erbB-2 and erbB-4 activate similar cellular signaling pathways during heart development. To date there is little information regarding the normal function(s) of erbB-3.

In mammary tissue, low levels of EGFR and erbB-2 protein can be detected in ductal epithelial and myoepithelial cells (47). The potent signaling activities of these two receptors in mammary tissue is underscored by the strong association of EGFR and erbB-2 overexpression in breast cancer. Indeed, EGFR and erbB-2 have been associated with poor prognosis in lymph node negative (66, 67, 68) and lymph node positive patients (3, 4, 69, 70), respectively. Moreover, overexpression of erbB-2 in the mammary epithelium of transgenic mice results in mammary tumor development with metastatic disease (9). Despite the strong association

of erbB-2 overexpression with the malignant proliferation of mammary tissue, the roles erbB-2 and other receptor tyrosine kinases play in normal mammary development remains unclear. Alternatively, one can address this issue by examining the affects of receptor tyrosine kinase regulating growth factors, namely members of the EGF family, on mammary development.

Recently the important role of HRG in mammary gland development has been investigated. Yang and co-workers (49) examined the affects of NRG on mammary gland morphogenesis in whole organ culture. In this experimental system, HRG stimulated lobuloalveolar development and the production of milk proteins. Strikingly, the developmental induction of HRGα expression, within the mammary mesenchyma, during pregnancy is strongly associated with a putative function in lobuloalveolar development and milk production. In mammary tumor cells, HRG appears to have a mitogenic effect (71, 72, 73, 74) or induce differentiation of mammary epithelium with the synthesis of milk proteins (30, 31, 72, 75). This variability in cellular responses may be a reflection of HRG isoform specific activities. Indeed, HRG β-isoforms have greater receptor binding affinity (31, 71) and stronger cellular growth effects (71, 72, 74) than α-isoforms. In addition, different cell lines may elicit different responses depending upon receptor expression heterogeneity and differing downstream signaling pathways generated by receptor activation.

Given the multifunctional roles of erbB-2 activating growth factors and erbB-2 transmodulation partners one strong prediction follows that the potentially complex, *in vivo* response of mammary epithelial cells to erbB-2 signaling is dependent upon target tissue expression of i) erbB-2 regulating growth factors and ii) erbB-2 heterodimerization partners. Clearly then, the essential role erbB-2 activation plays in normal and malignant mammary epithelial development can only be clarified when the local co-expression of all known EGFR family members and each activating growth factor is deduced. To this end, I have employed RNase protection assays (RNPA) to determine the expression of EGFR family members and their agonists during normal mouse mammary gland development. In addition, the physiological presentation of relevant receptors and ligands

will be altered during mouse mammary gland development through the use of transgene expression of dominant negative receptors and mammary gland implants containing recombinant or synthesized growth factors, respectively. The results from these experiments will provide important information regarding the regulation of erbB-2 signaling during normal mammary gland development. This information will be utilized to elucidate the role erbB-2 plays in the development of breast cancer and eventually provide therapy strategies which down-regulate aberrant erbB-2 signaling.

Results and Discussion

Receptor and Ligand Expression

The majority of mammary gland development occurs post-natally where mammary epithelium progress through stages of proliferation in the virgin, differentiation during pregnancy, lactation, and apoptosis in the weaned mammary gland. Regulated expression of receptors and/or ligands within mammary epithelium may provide important functional information. To determine the expression levels of each EGFR family member I employed RNPA to analyze total RNA from staged mouse mammary glands. Briefly, total RNA was isolated from the number 4 inguinal mammary gland of Balb/C female mice using the GITC extraction method (76). Specific riboprobes synthesized from receptor cDNA (generously provided by Gerald Chu)(77) were hybridized to 50 μg of total RNA and the digested hybrid was analyzed by acrylamide gel electrophoresis. Riboprobe synthesized from mouse cyclophilin cDNA was used as an internal control.

A typical RNPA result is shown in Figure 1A. In this example both erbB-2 and erbB-3 are expressed throughout the different stages of mammary gland development. When all four receptors are analyzed and the results quantitated it is clear that each receptor is expressed at relatively constant levels during normal mammary gland development (Figure 1B). These results indicate that expression of EGFR family members is not regulated during normal mouse mammary gland development.

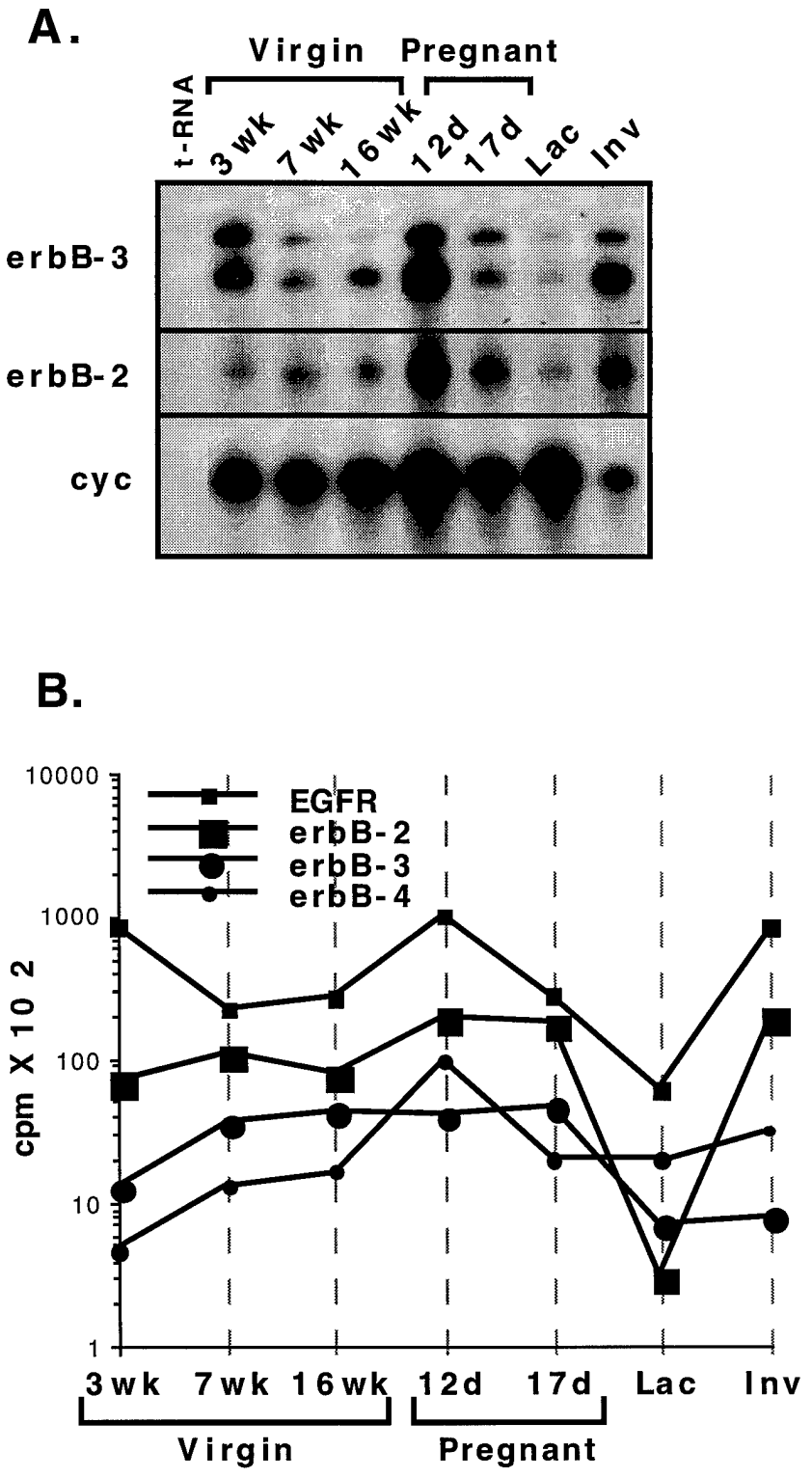

Figure 1. RNPA of staged mouse mammary gland RNA using riboprobes representing each EGFR family member. Abbreviations are: Lac, 10 days lactation; Inv, 7 days involution.

Initial analysis of EGF family members by RNPA yielded surprisingly different results. For example, in Figure 2A and B expression of AR appears to be regulated and only expressed at high levels in virgin mammary glands whereas similar experiments performed by Yang et. al. (1995)(49) indicate that HRGα expression is regulated during pregnancy. These later results are reproduced in Figure 2B.

Thus it appears that regulation of growth factor expression may drive normal mammary gland development through stages of proliferation and differentiation. To test this hypothesis slow release pellets containing HRG were implanted within virgin mammary glands in an attempt to induce epithelial differentiation.

Mammary Gland Implants Containing HRG

Based upon receptor and ligand expression studies I propose that regulated ligand expression may drive mammary gland development. To test this hypothesis I implanted pellets containing HRG within the mammary glands of virgin female mice. I predict that HRG would induce epithelial differentiation within virgin mammary glands whereas control implants containing TGFα would not induce differentiation. The results from these experiments have been published and reprints are included in the Appendix (78). Briefly, we found that implants containing HRG or TGFα induced epithelial proliferation and lobuloalveolar development; however, only implants containing HRG resulted in the accumulation of secretory products within the lumens of lobuloalveolar structures. In subsequent experiments I stained the HRG induced lobuloalveolar structures histologically with an antibody against mouse caseins. If HRG induces differentiation I would expect to see expression of milk proteins including casein. The results from this experiment are shown in Figure 3. Hematoxylin/eosin (H + E) staining of paraffin imbedded HRG treated mammary glands demonstrate the presence of secretory products (SP) within ductal lumens (Figure 3; upper panel). These secretory products stained positive for mouse casein (Figure 3; lower panel) but did not stain with a negative control antiserum (data not shown).

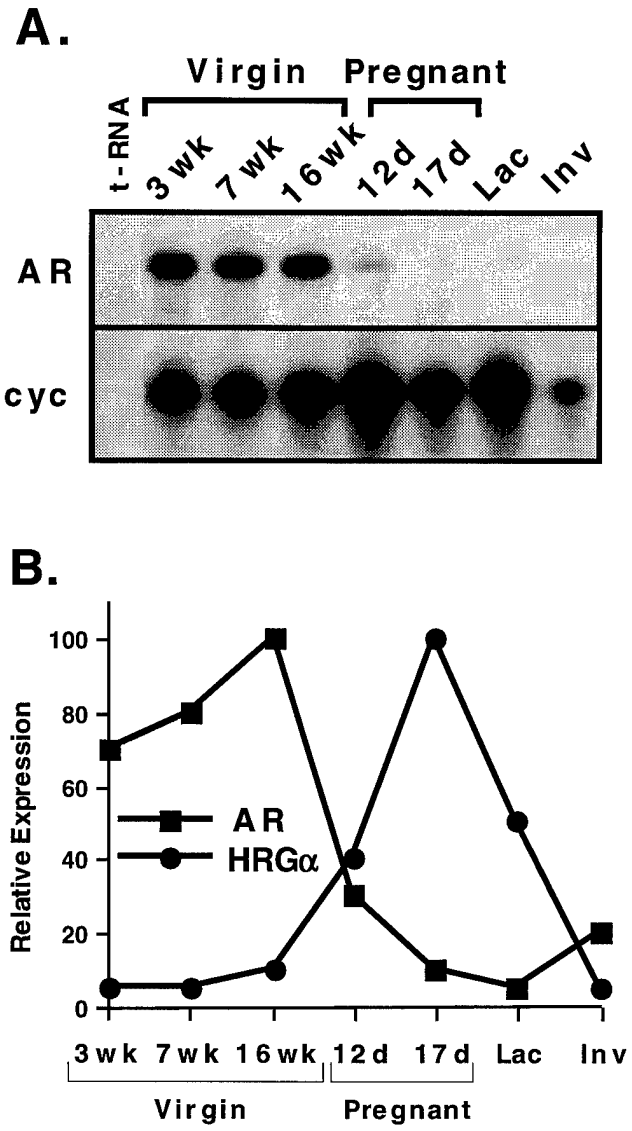

Figure 2. RNPA of staged mouse mammary gland RNA using a riboprobe representing AR. Data for HRGα was published elsewhere (49).

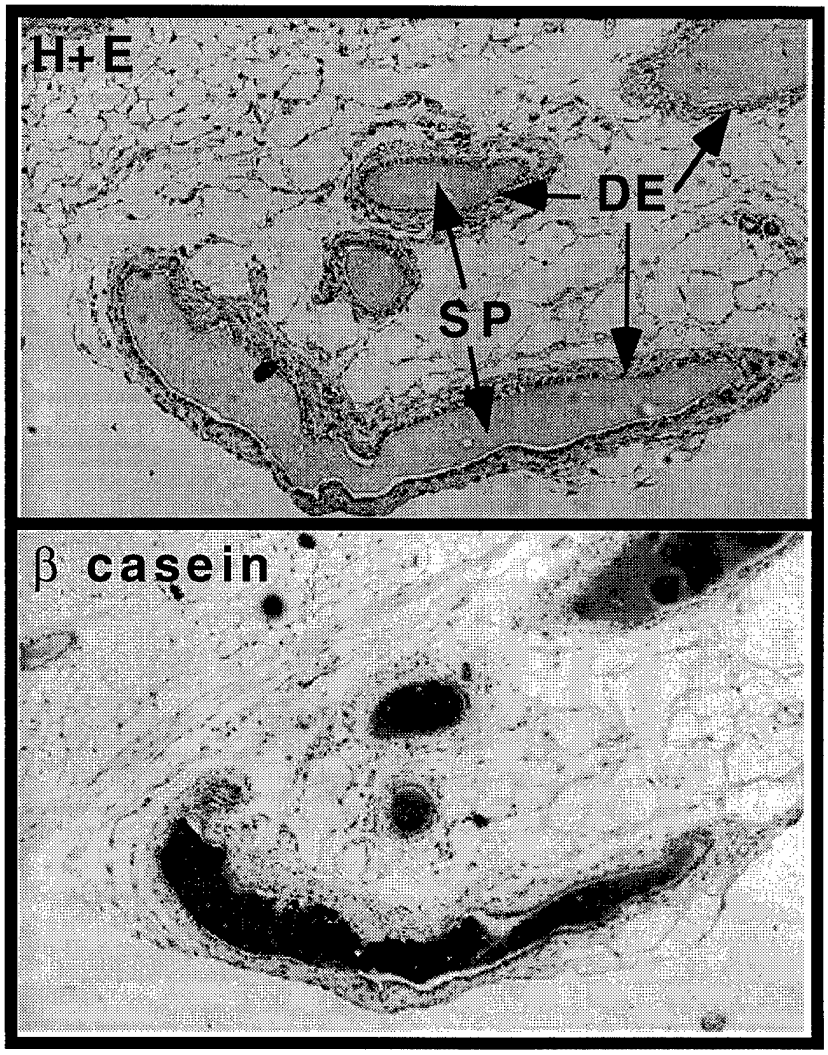

Figure 3. Mammary glands implanted with pellets containing HRG were excised, embedded in paraffin, sectioned and stained with hematoxylin/eosin (upper panel) or stained histologically with antibody specific for mouse casein (lower panel).
Abbreviations: DE, ductal epithelium; SP, secretory products.

These results provide strong evidence that HRG is involved in mammary epithelial differentiation. Moreover, the continuous expression of EGFR family members suggests that regulated ligand expression is responsible for developmental changes within the mammary gland. I will pursue additional experiments to test this hypothesis.

Transgene Expression of Dominant Negative ErbB-2

To determine the functions of the EGFR family during normal mammary gland development I attempted to eliminate receptor signaling within the mammary gland. The most direct method to nullify receptor signaling *in vivo* would be to perform gene knockouts in mouse embryonic stem cells and analyze the mice derived from these cells. However, gene knockouts of three receptors analyzed to data each results in an embryonic lethal phenotype (59, 64, 65). I therefore took an alternative approach and expressed a dominant negative erbB-2 receptor (79, 80, 81) within the mouse mammary gland as a transgene. The dominant negative receptor lacks the cytoplasmic C-terminus including putative phosphorylation sites and the receptor kinase. To drive expression within the mammary gland the mutant receptor was placed downstream of the mouse moloney tumor virus (MMTV) LTR. Preliminary results indicate that successful transgene transmission has occurred and mammary gland expression of the mutant receptor was observed in at least one founder. Analysis of the effects of this transgene on normal mammary gland development is in progress.

Conclusions

The long-term goal of the research described in this report is to determine the role erbB-2 plays in breast cancer development. To address this issue I will analyze the functions of EGFR family members and their ligands during normal mammary gland development and relate these findings to normal and aberrant erbB-2 activity.

In this communication, the expression levels of EGFR members was determined by RNPA. Receptor expression was unregulated and was detected continuously during mammary gland development. Expression of the ligands AR and HRG on the other hand was regulated. Interestingly, AR

expression was only detected within mammary glands of virgin animals whereas HRG expression was only detected during pregnancy. These results suggested that ligand expression may drive receptor signaling and thus mammary gland development. As all four receptors were present in both virgin and pregnant mammary glands I predicted that altered ligand presentation may alter normal mammary gland development. To test this hypothesis I implanted pellets containing HRG within the mammary glands of virgin female mice. The HRG implants induced differentiation of virgin mammary epithelium forming lobuloalveolar structures with the accumulation of milk proteins. This result provides strong evidence that HRG is in fact involved in mammary epithelial differentiation and can drive differentiation *in vivo* when all four EGFR family members are expressed.

Further experiments will be designed to determine which receptor(s) are involved in HRG induced differentiation. Presumably erbB-3 and /or erbB-4 must be involved as these receptors directly bind HRG (40, 41). EGFR and erbB-2 may also play an important role in the HRG differentiation pathway as these receptors can be activated by HRG through erbB-3 and erbB-4 (40, 41, 56). When the HRG differentiation pathway is deciphered I will use these findings to develop therapeutic strategies to downregulate erbB-2 signaling in breast cancer by inducing differentiation of breast tumor cells.

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Appendix

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Heregulin Induces *in Vivo* Proliferation and Differentiation of Mammary Epithelium into Secretory Lobuloalveoli[1]

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Abstract

Mammary gland development and differentiation is mediated through the combined activities of systemic hormones and locally synthesized growth factors. To determine the *in vivo* response of mammary epithelium to heregulin (HRG), we implanted Elvax pellets containing HRGα or HRGβ within the mammary glands of prepubescent female mice in the presence or absence of exogenous estradiol and progesterone (E/P). Mice treated in the same way with transforming growth factor α (TGF-α) were included as a positive control. Each growth factor treatment induced epithelial ductal branching in the presence or absence of E/P. In the absence of E/P, HRGβ did not affect terminal end bud formation, mammary epithelium branching, or ductal migration. In contrast, TGF-α and HRGα induced ductal branching and HRGα induced ductal migration in the absence of E/P. The overall mammary response to growth factors was potentiated by the concomitant presence of E/P. In every case, the *in vivo* mammary epithelial responses to HRGα were more robust than TGF-α. Limited lobuloalveolar development was also observed in growth factor-treated mammary glands when E/P was present. Histological examination of growth factor-induced lobuloalveoli revealed secretory products within the lumen of HRGα and HRGβ lobuloalveoli. TGF-α-induced lobuloalveoli lacked similar secretory products.

Introduction

Mammary gland development is unusual in that the vast majority of growth and differentiation occurs postnatally. In the prepubescent mouse, mammary ductal structures emanating from the nipple terminate in large bulbous structures referred to as TEBs.[3] With the onset of puberty, steroid hormones function as potent mitogens of TEB. This rapidly dividing cell population is responsible for ductal growth and branching during expansion of the mammary gland. During pregnancy, an additional pronounced growth cycle results in increased ductal branching and lobuloalveolar development. The lobuloalveoli terminally differentiate into milk-producing structures, and the extensive lobuloalveoli completely fill the interductal spaces during lactation (1, 2). These developmental processes are regulated through a complex series of events requiring the activities of both intraglandular and systemic hormones/growth factors (3–5). The steroid hormones estrogen and progesterone are major players in these developmental processes. However, the exact mechanisms underlying steroid hormone growth effects are not known and may involve a combination of direct effects and/or stimulation of growth factors which in turn mediate mammary gland development in a juxtacrine or autocrine fashion. Indeed, estradiol stimulates mammary epithelial expression and/or secretion of several EGF family members (6–10), and these growth factors have several important functional roles during mammary gland development (4, 5, 11).

Normal breast tissue expresses several EGF family members including EGF (12), TGF-α (12–16), amphiregulin (16–18), cripto-1 (16–18), and HRG (19). In addition, mammary gland expression of all four EGFR family members identified to date (*e.g.*, EGFR, erbB-2/HER-2/neu, erbB-3, and erbB-4) has been reported (19–22). A substantial body of evidence suggests that the EGF family of growth factors and their cellular receptors play an important role in both normal and malignant mammary gland development (4, 5, 11, 23–27). Most recently, the function of HRG in mammary gland development has been investigated. In mammary tumor cells, HRGs appear to have a mitogenic effect (28–31) or induce differentiation of mammary epithelium with the synthesis of milk proteins (30, 32–34). Yang *et al.* (19) examined the affects of HRG on mammary gland morphogenesis. In whole-organ culture, HRG stimulates lobuloalveolar development and the production of milk proteins. A putative role for HRG in lobuloalveolar development and milk production is further supported by the following observations; HRGα is expressed within the mammary mesenchyme adjacent to lobuloalveolar structures, and HRGα expression is regulated during mammary gland development and is only expressed during pregnancy (19). Therefore, HRG appears to be a po-

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[3] The abbreviations used are: TEB, terminal end bud; EGF, epidermal growth factor; EGFR, EGF receptor; HRG, heregulin; TGF-α, transforming growth factor α; E/P, estradiol and progesterone; RP-HPLC, reverse phase high pressure liquid chromatography.

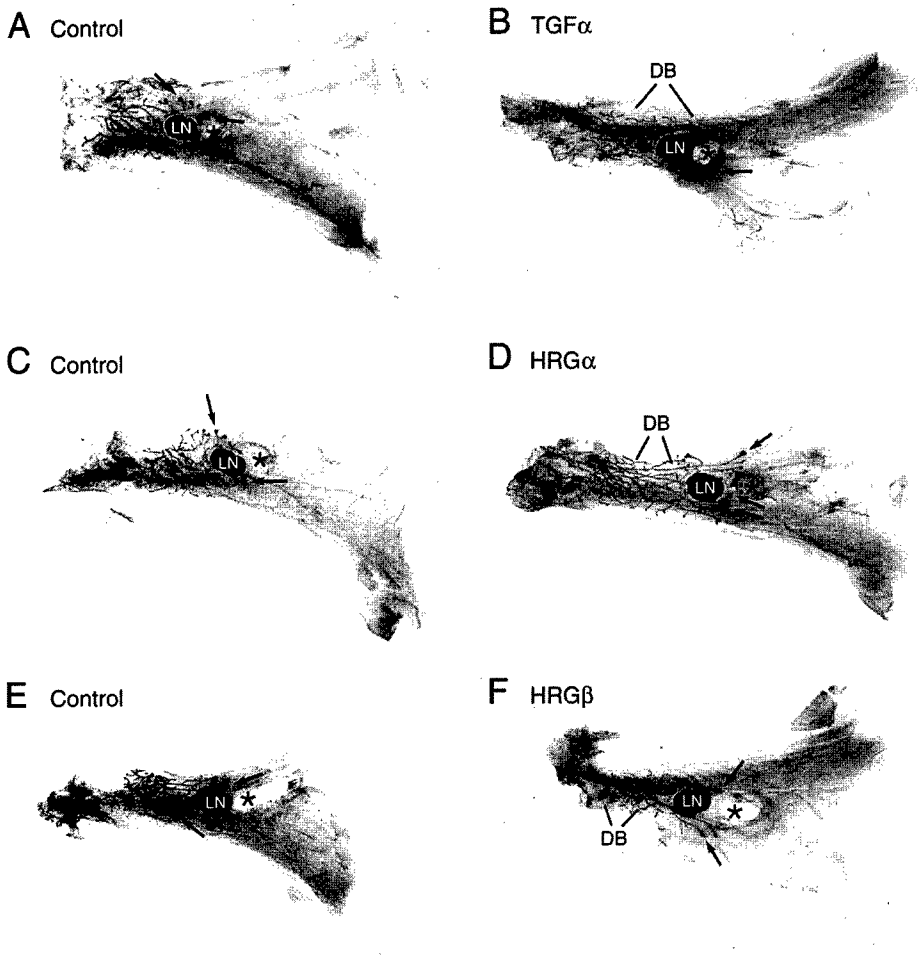

Fig. 1. Effect of growth factor treatment on mammary gland morphology in the absence of estradiol and progesterone. Control Elvax pellets and pellets containing growth factor were implanted within the surgically exposed number 4 inguinal mammary fat pad of 30-day-old female BALB/c mice. Implants were positioned (*) immediately anterior to the central lymph node (*LN*). Mice were sacrificed 3 days following implant insertion, and excised mammary glands were processed using the whole-mount procedure and stained with carmine (see "Materials and Methods"). Terminal end buds are indicated by *arrows*, and regions of growth factor-induced ductal branching (*DB*) are indicated. Implants contained 10 μg of TGF-α (*B*), 5 μg of HRGα (*D*), and 10 μg of HRGβ (*F*). Contralateral control for each sample is represented (*A*, *C*, and *E*).

tent and developmentally important mammary epithelial growth factor.

Despite these recent observations, the role of HRG in mammary gland morphogenesis *in vivo* has not been investigated. To address this issue, we have used a mouse model system to examine the direct effects of HRG on mammary epithelium under conditions similar to those where this growth factor normally functions. The mammary glands of prepubescent female mice were surgically implanted with Elvax pellets containing recombinant HRGα, HRGβ, or TGF-α in the presence or absence of steroid hormones. All three growth factors possessed differing levels of epithelial mitogenic activity *in vivo*. In addition, in the presence of steroid hormones, each growth factor induced epithelial differentiation into lobuloalveolar structures. However, only the HRG-treated lobuloalveoli underwent terminal differentiation, resulting in the lumenal accumulation of secretory products. Taken together, these experiments offer the first *in vivo* evidence for a role of HRG in mammary epithelial development and terminal differentiation into milk protein-secreting lobuloalveolar structures.

Results

HRG Induces Ductal Branching *in Situ*. HRG induces pleiotropic responses in cultured mammary epithelial cells (19, 28, 29, 31, 33–35); however, the *in vivo* response of mammary epithelium to this family of growth factors has not been investigated. As a first step toward identifying a biological role for HRG in mammary ductal morphogenesis, we surgically implanted slow-release pellets containing varying amounts of HRGα or HRGβ within the developing mammary fat pad of virgin female mice. HRGα and HRGβ are splice variants that possess differing EGF domains (36). Pellets lacking growth factor were inserted into the contralateral fat pad as a negative control. Previously, TGF-α has been shown to induce ductal branching and lobuloalveolar development in a similar experimental system (37) and was, therefore, included as a positive control in our experiments. The mice were sacrificed 3 days after implant insertion, and whole mounts of the mammary glands were examined for ductal morphogenesis and lobuloalveolar development.

When compared to contralateral controls, each growth factor induced ductal branching within the treated mammary gland (Figs. 1 and 2). Responses to growth factors in the

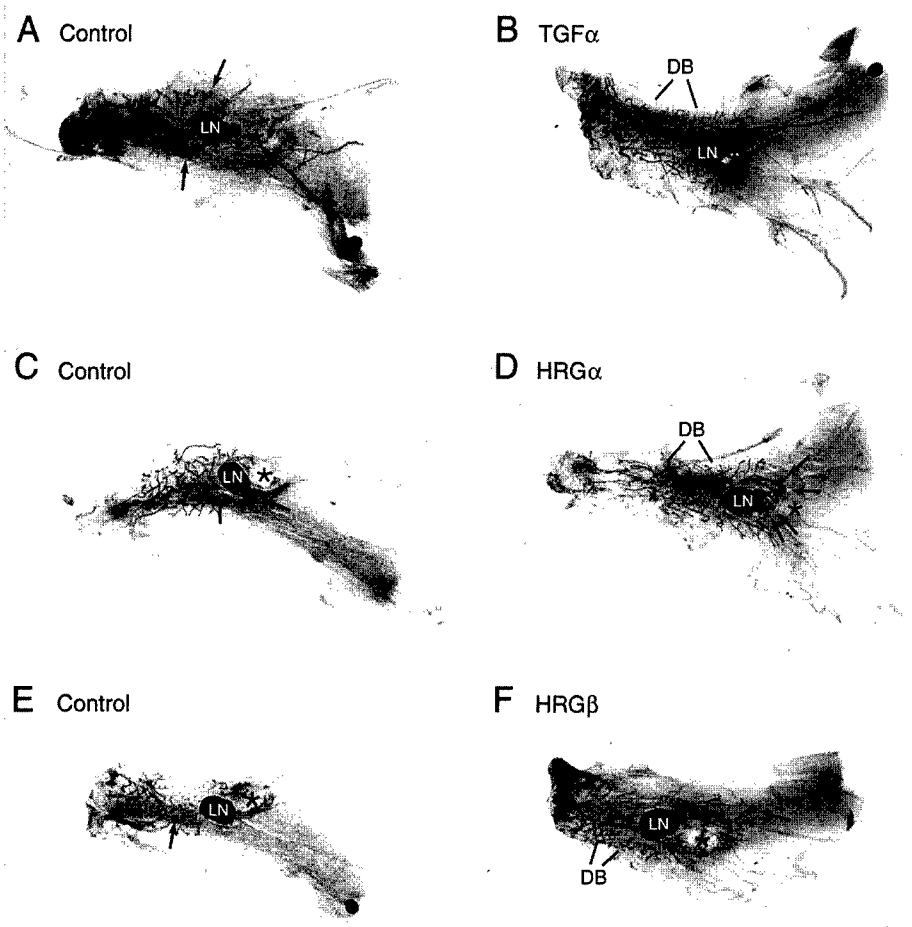

Fig. 2. Effect of growth factor treatment on mammary gland morphology in the presence of estradiol and progesterone. Control Elvax pellets containing 10 μg of 17 β-estradiol and 1 mg of progesterone (E/P) and pellets containing growth factor with E/P were implanted within the surgically exposed number 4 inguinal mammary fat pad of 30-day-old female BALB/c mice. Implants were positioned (*) immediately anterior to the central lymph node (*LN*). Mice were sacrificed 3 days following implant insertion, and excised mammary glands were processed using the whole-mount procedure and stained with carmine (see "Materials and Methods"). Terminal end buds are indicated by *arrows*, and regions of growth factor-induced ductal branching (*DB*) are indicated. Implants contained 10 μg of TGF-α (*B*), 5 μg of HRGα (*D*), and 10 μg of HRGβ (*F*). Paired contralateral control for each sample is represented (*A, C,* and *E*).

presence or absence of E/P were maximal with pellets containing 10 μg of TGF-α or HRGβ or 5 μg of HRGα (data not shown). These growth factor concentrations were used in all subsequent experiments. In the absence of E/P, each growth factor induced ductal branching in the region posterior to the central lymph node (Fig. 1, compare *panels A* to *B*, *C* to *D*, and *E* to *F*). However, differences between each growth factor could be identified. For example, the ductal branching observed in HRGβ-treated glands (Fig. 1*F*) was less extensive than glands treated with either TGF-α (Fig. 1*B*) or HRGα (Fig. 1*D*). In addition, where TGF-α and HRGβ treatment appeared to inhibit TEB formation (Fig. 1, *B* and *F*, respectively), HRGα not only induced TEB proliferation but also increased ductal migration anterior to the central lymph node (Fig. 1*D*).

Treatment of control mammary glands with E/P alone resulted in a slight increase in ductal diameter (compare Fig. 1*A* to Fig. 2*A*). Moreover, the mammary response to growth factors was potentiated by the presence of E/P because ductal branching induced by each growth factor was more pronounced in the presence of E/P (compare Fig. 1, *B, D,* and *F,* to Fig. 2, *B, D,* and *F,* respectively). The mammary epithelial responses to implants containing TGF-α (Fig. 2*B*) and HRGβ (Fig. 2*F*) were similar because both growth factors inhibited TEB formation. In contrast, HRGα induced TEB formation, and the overall epithelial response to HRGα was more robust (Fig. 2*D*) than either TGF-α (Fig. 2*B*) or HRGβ (Fig. 2*F*).

The extent of ductal branching, ductal growth, and TEB formation induced by each growth factor in the presence and absence of E/P was quantitated. Data from 10 mice, for each experimental condition, was subjected to statistical analysis. Due to high variability among mice, each quantitated parameter was normalized to the contralateral control within an individual animal. Although each growth factor induced ductal branching (Figs. 1 and 2), branching induced by HRGβ was statistically significant only in the presence of E/P (Fig. 3). In general, HRGα appeared to induce a more robust and pleiotropic response within treated mammary glands than either TGF-α or HRGβ. Indeed, ductal branching was more extensive in HRGα-treated glands whether in the presence or absence of E/P (Fig. 3). Moreover, whereas TGF-α and HRGβ appeared to slightly inhibit or had no affect on TEB formation, HRGα induced TEB proliferation in the presence of E/P (Fig. 3). Moreover, HRGα was the only growth factor to significantly increase ductal length within treated mammary glands

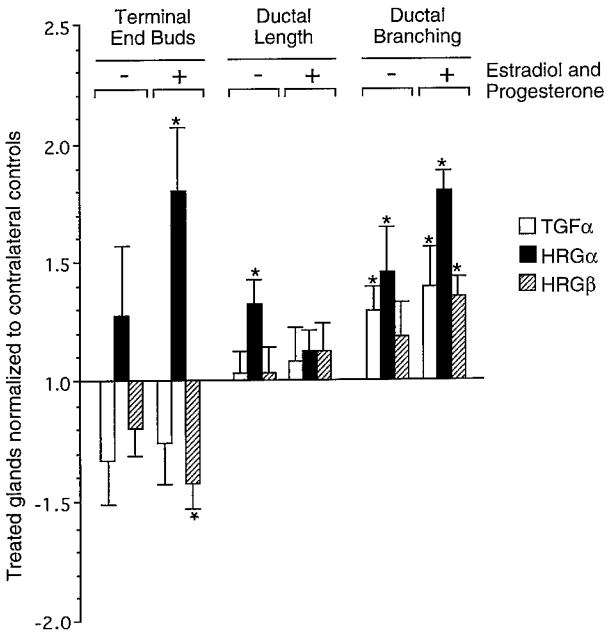

Fig. 3. Effects of growth factors on mammary epithelium. Ten 30-day-old female BALB/c mice were implanted with growth factor pellets at the growth factor's concentration of maximal response in the absence (−) or presence (+) of 10 μg of estradiol and 1 mg of progesterone. Mice were sacrificed 3 days following implant insertion, and excised mammary glands were processed using the whole-mount procedure and stained with carmine (see "Materials and Methods"). The number of TEBs, the ductal length of the longest duct per fat pad, and the number of ductal branches was determined for each treated and contralateral control gland. Treated glands were normalized to their contralateral controls, and the means plus 1 SD (*bars*) are represented above. Growth factor-induced phenotypes significantly different from contralateral controls (paired student *t* test; $P < 0.01$) are indicated (*). Implants contained 10 μg of TGF-α, 5 μg of HRGα, and 10 μg of HRGβ.

(Fig. 3). With the exception of increased ductal length induced by HRGα in the absence of E/P, the concomitant presence of E/P appeared to potentiate the mammary epithelial response to each growth factor tested (Fig. 3).

HRG Induces Mammary Lobuloalveolar Development and the Accumulation of Lumenal Secretory Products *in Vivo*. Whole mounts prepared from growth factor-treated mammary glands revealed extensive epithelial ductal branching. We next examined the terminal ducts within treated and control mammary glands for evidence of lobuloalveolar structures. As expected, untreated glands or glands treated with E/P alone did not develop lobuloalveolar structures. In contrast, a limited extent of lobuloalveolar development was observed in a majority of mammary glands treated with growth factor (Fig. 4, *A-C*). Growth factor-induced lobuloalveoli required the concomitant presence of E/P because similar structures were not observed in glands treated with growth factors alone. Consistent with previous results, the effect of HRGα was more robust than either TGF-α or HRGβ. HRGα induced lobuloalveolar development in 77% of treated mammary glands, whereas TGF-α and HRGβ induced lobuloalveoli in 39 and 46% of treated glands, respectively (13 glands were examined for each treatment). Histological examination of growth factor-induced lobuloalveoli revealed numerous epithelial buds typical of these structures (Fig. 4, *D-F, arrows*). In addition, HRGα- and HRGβ-induced lobuloalveoli exhibited accumulation of lumenal secretory products (Fig. 4, *E* and *F*), which stained positive for β-casein by immunohistochemistry (data not shown). Similar accumulations were not observed in TGF-α-induced lobuloalveoli (Fig. 4*D*).

Discussion

Mammary gland development involves a complex and highly regulated sequence of postnatal events. Recently, expression of an EGF-related subfamily of growth factors termed the neu differentiation factors or HRGs was detected *in vivo* within connective tissue juxtaposed to fully differentiated, milk-secreting lobuloalveoli (19). To determine if HRG plays a role in mammary epithelial growth and/or differentiation *in vivo*, we inserted slow-release pellets containing HRG within mammary glands of prepubescent mice and analyzed the *in vivo* response of mammary epithelium to these growth factors. We found that HRGα and HRGβ induced epithelial branching and differentiation into lobuloalveolar structures, as does a related growth factor, TGF-α. However, histological examination of TGF-α- and HRG-induced lobuloalveoli revealed a striking difference; HRGα and HRGβ stimulated the accumulation of lumenal secretory products, including the milk protein β-casein, within treated lobuloalveoli. TGF-α-induced lobuloalveoli lacked similar lumenal accumulations. These results suggest that HRG can induce terminal differentiation of mammary epithelium *in vivo* into milk protein-secreting lobuloalveolar structures.

The epithelial response to growth factor implants was potentiated by the concomitant presence of estradiol and progesterone. Indeed, lobuloalveoli were only observed in the presence of these steroid hormones. A similar requirement of estradiol and progesterone for EGF- and HRG-induced lobuloalveoli in mammary organ culture has been reported (4, 19, 38). Some evidence suggests that the requirement of exogenous steroid hormones may also reflect strain differences. For example, TGF-α implants induce lobuloalveoli development in CH3/HeN mice in the absence of exogenous estradiol and progesterone (37). In contrast, our results indicate that induction of lobuloalveoli by TGF-α or HRG in BALB/c mice requires the concomitant presence of exogenous estradiol and progesterone. Although we did not perform experiments to determine if estradiol or progesterone alone could augment mammary responses to growth factors, substantial evidence indicates that both estradiol and progesterone have independent proliferative effects on mammary epithelium. Furthermore, co-administration of these hormones enhances independent proliferative effects (3). Using mice carrying a null mutation in the progesterone receptor, Lydon *et al.* (39) demonstrated the *in vivo* requirement of progesterone in ductal epithelium proliferation and lobuloalveoli differentiation. Thus, it seems probable that progesterone contributes to the growth factor-induced lobuloalveolar development observed in our experiments. Although estradiol is considered to be the steroid hormone most directly involved in mammary epithelial proliferation (3), the exact role of estradiol in mammary development is poorly defined. The

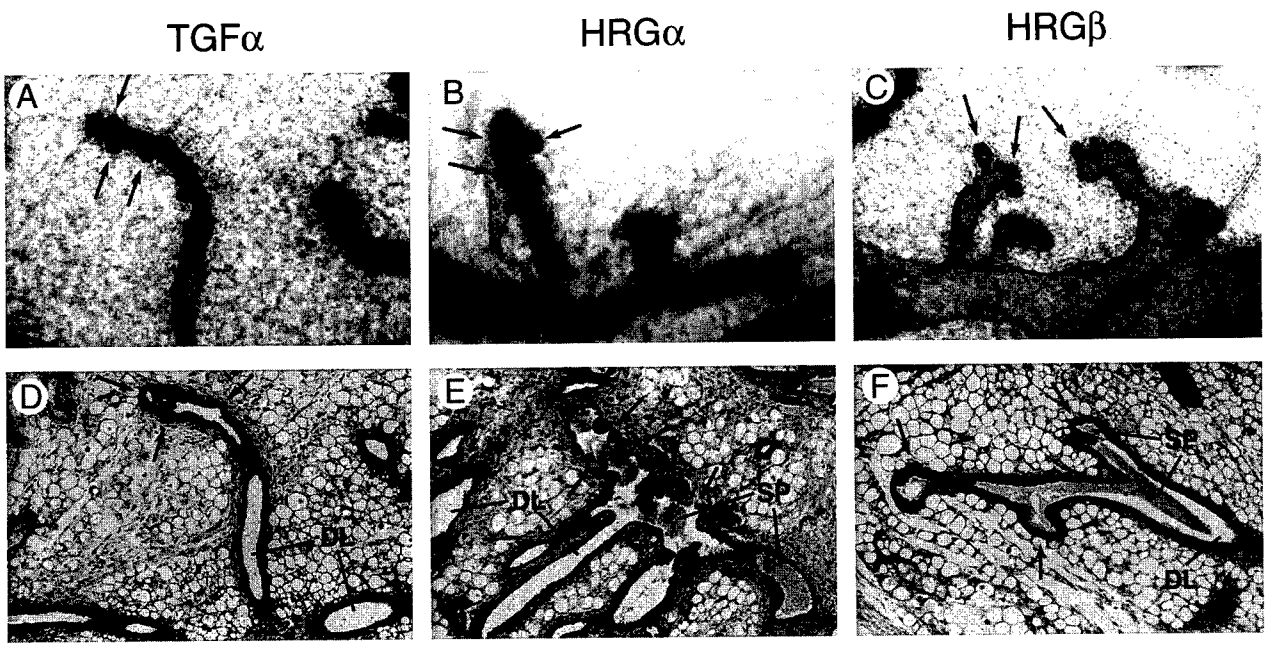

Fig. 4. Growth factor-induced lobuloalveolar development in prepubescent female mice. Elvax pellets containing growth factor with 10 μg of 17 β-estradiol and 1 mg of progesterone were implanted within the surgically exposed number 4 inguinal mammary fat pad of 30-day-old female BALB/c mice. Mice were sacrificed 3 days following implant insertion, and excised mammary glands were processed using the whole-mount procedure and stained with hematoxylin (see "Materials and Methods"). Photomicrographs of paraffin sections revealed lobuloalveolar structures in growth factor-treated glands (*A-C*). *Arrows* indicate epithelial buds typical of these structures. Histological examination of paraffin-embedded lobuloalveoli stained with H&E (*D* and *E*) revealed secretory products (*SP*) within the ductal lumens (*DL*) of HRGα- and HRGβ-treated mammary glands (*E* and *F*, respectively). Implants contained 10 μg of TGF-α (*A* and *D*), 5 μg of HRGα (*B* and *E*), and 10 μg of HRGβ (*C* and *F*).

reason for this lack of clarity lies in the fact that in addition to independent proliferative effects, estradiol also induces mammary expression of several growth factors, including the EGF family members EGF (7), TGF-α (6, 7, 40), and amphiregulin (9, 41). Mammary gland expression of these EGF family members may directly affect mammary development and thereby augment the epithelial response to growth factor implants observed in our experiments.

The qualitative and quantitative differences in mammary epithelial responses to TGF-α and HRG can be explained through functional differences of the two growth factors. Histochemical analysis of mouse mammary glands reported elsewhere revealed dramatic differences in the cellular localization and expression of these growth factors. Mammary gland expression of TGF-α was detected during each epithelial developmental stage with the exception of lactation, and immunostaining within the cap cell layer of the TEB and epithelial cells of subtending ducts was observed (12). In contrast, expression of HRG is induced during pregnancy within the connective tissue adjacent to ductal and lobuloalveolar structures (19). Differing mammary epithelial responses to TGF-α and HRG may also reflect the activation of different signaling tyrosine receptor kinases within these cellular populations. TGF-α binds directly to the EGFR (26) and can activate erbB-2 (42), erbB-3, and erbB-4,[4] presumably through a ligand-driven receptor cross-phosphorylation mechanism (43, 44) also referred to as "transmodulation" (24, 45). Similarly, HRG binds directly to erbB-3 (46, 47) and erbB-4 (47) and can drive the activation of EGFR and erbB-2 (46–48). Evidence from *in vitro* experiments indicates that cellular responses to signaling by this family of receptors can be radically different, depending upon both the transmodulation partner and the activating growth factor (48–51). Therefore, one prediction follows that signaling by EGFR family members *in vivo* would also induce a diversity of cellular responses that are dependent upon the activating growth factor. Cellular responses to HRG *in vivo* appear to be regulated primarily but not exclusively through erbB-2 signaling. Disruption of HRG or erbB-2 in transgenic mice results in a similar embryonic lethal phenotype characterized by nearly identical heart malformations and neural crest development defects (52, 53). Moreover, expression patterns within the developing rhombencephalon suggest that a HRG: erbB-2 autocrine or paracrine signaling relationship has been disrupted in these mice (52, 53). These observations further support a direct relationship between HRG and erbB-2 signaling. A similar relationship may mediate HRG activity in mammary epithelium, and we are presently designing experiments to examine this possibility.

In our experiments, the *in vivo* response of mammary epithelium to HRGα was more robust than HRGβ. This result was surprising because *in vitro* experiments consistently identify HRGβ as the more potent growth factor (28, 30, 31, 33). However, we used chemically synthesized and bacterial recombinant peptides in our experiments, which may not

[4] D. J. Riese II, E. Kim, G. Allison, S. Buckley, M. Klagsbrun, G. D. Plowman, and D. F. Stern. J. Biol. Chem., in press.

represent the complete activities of full-length HRG protein. Alternatively, the enhanced mammary response to HRGα may reflect a physiological role for HRGα and not HRGβ in mammary gland development. Indeed, only HRGα isoforms are expressed in the mammary gland, and this expression is induced during pregnancy (19). Thus, the HRGα expression pattern strongly correlates with the *in vivo* function identified in this communication. Our experiments provide the first demonstration of an important *in vivo* role for HRG in mammary epithelium proliferation and differentiation into secretory lobuloalveoli. In conclusion, we propose that HRGα is the physiologically relevant HRG isoform expressed within the developing mammary gland, and HRGα plays an important role in the differentiation of mammary epithelium into milk-secreting lobuloalveoli.

Materials and Methods

Plasmid Construction. The human HRG β1 cDNA fragment corresponding to residues 177–244 (54) was subcloned into the pNB261 bacterial expression vector as follows. Poly(A) mRNA was isolated from cultured human MDAMB231 cells (American Type Culture Collection) by use of the Fast Track mRNA isolation kit (InVitrogen), according to the manufacturer's instructions. The HRG β1 cDNA fragment corresponding to residues 177–244 was amplified by a 30-cycle reverse transcription-PCR procedure using the RNA Gene Amp kit (Perkin-Elmer Corp.) and the primers incorporating 5′ *Eco*RI (sense 5′-CGCGAATTCTATGAGCCATCT-TGTAAAATGTGC) and *Hind*III (anti-sense 5′-CGCGAAGCTTAGTA-CAGCTCCTCCGCCTCCAT) linkers. The 204-bp amplified fragment was digested with *Eco*RI/*Hind*III and inserted into the same sites of the Bluescript vector pCRII (Stratagene). The nucleotide sequence of the 204-bp insert was confirmed by use of an Applied Biosystems Automated Sequencer using standard methods. The sequenced 204-bp insert was excised from pCRII by digestion with *Eco*RI/*Hind*III and subcloned downstream of the trp-inducible promoter using the same restriction sites of the pNB261 expression vector (construct pHerβ1ST). The sequence of the 204-bp human HRG β1 insert was confirmed as described above.

Expression and Purification of Human Recombinant HRG β1 (177–244). For large scale fermentation and expression of HRG β1 (177–244), pHERβ1ST was transformed into the *Escherichia coli* strain GE81. Bacterial cells from a 10-liter fermentation in modified M9 medium (55) were harvested by centrifugation, and expression was induced by resuspending the bacteria pellet into fresh modified M9 medium lacking tryptophan. After an induction period of 4 h, a total of 69 g of cell paste was recovered by centrifugation. Expression of the predicted 7000-Da product peaked at 3 h postinduction. A 25-g bacterial pellet was resuspended into 50 ml of lysis buffer [20 mM Tris (pH 8.0), 40 mM NaCl, 0.25 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride] and passed through a French press twice. The lysate was centrifuged for 45 min at 12,000 rpm, and the pellet was resuspended into 20 ml of lysis buffer. Fifteen mg of hen egg white lysozyme (Sigma Chemical Co.) was added, and the mixture was incubated at room temperature for 20 min. Three hundred μl of 1 mg/ml DNase and 800 μl of 100 mM $MgCl_2$ were added, and the mixture was incubated at room temperature for an additional 15 min. The suspension was centrifuged for 15 min at 12,000 rpm, and the final inclusion body pellet was washed twice with 1.0% NP40 and once with ddH_2O and lyophilized to dryness to yield 500 mg of dried inclusion bodies. A suspension of 10 mg of inclusion bodies in 2.0 ml of 50 mM Tris (pH 6.0), 6 M guanidinium hydrochloride, and 200 mM DTT was heated at 37°C for 2 h and filtered through a Costar 3.5-μm spin-filter; then the filtrate was diluted into 100 ml of folding buffer [50 mM Tris (pH 8.6), 1 M urea, 1.5 mM glutathione, 0.75 mM glutathiol, and 10 mM methionine] and stirred for 5 days at 4°C. The folded, oxidized protein was isolated by RP-HPLC on a VYDAC C-4 reverse phase column using an acetonitrile/ddH_2O/0.1% trifluoroacetic acid gradient. The isolated protein was homogeneous by RP-HPLC and capillary electrophoresis, and was composed of 206 μg by amino acid analysis. The protein exhibited a mass of 7877.8 Da by electrospray mass spectrometry [theoretical mass for oxidized HRG β1 (177–244) is 7878.1 Da].

Growth Factors. HRGα 177–228 (HRGα 52) was synthesized on an Applied Biosystems 430A peptide synthesizer using standard *tert*-butyloxycarbonyl chemistry protocols provided by the manufacturer (version 1.40; *N*-methylpyrolidone/hydroxybenzotriazole). Peptide was purified by RP-HPLC, characterized by electrospray mass spectroscopy, and analyzed for disulfide bonding as described previously (56). Peptide quantities were determined by amino acid analysis. Human recombinant TGF-α was purchased from Collaborative Biomedical Products.

Implant Preparation. Growth factor peptides and steroid hormones were encapsulated within Elvax pellets essentially as described elsewhere (57). Briefly, a lyophilized mixture containing growth factor peptide and, where indicated, the steroid hormones 17-β-estradiol (10 μg; Sigma) and progesterone (1 mg; Sigma) was suspended in 25 μl of Elvax (generously donated by Elf Atochem, Philadelphia, PA) dissolved previously in dichloromethane (15% w/v). The entire suspension was transferred to an Eppendorf tube, snap-frozen in liquid nitrogen, and dried under vacuum. The dried Elvax pellet was compressed between tweezers such that the final pellet was ~1 mm in diameter and weighed 2–3 mg.

Surgical Implantation. Thirty-day-old virgin female BALB/c mice (Charles River) were used in all experiments. Mice were anethesized with an i.p. injection of 250–350 μl of avertin [20 mg/ml 2,2,2-tribromoethanol (Aldrich) in saline]. The number 4 inguinal mammary fat pad was surgically exposed, and a 2-mm incision was made through the mammary fat pad outer membrane immediately anterior to the central lymph node. The Elvax pellet was placed within the incision and immobilized under the mammary fat pad outer membrane. Control pellets lacking growth factor were inserted into the contralateral number 4 inguinal mammary fat pad. The wounds were closed using surgical staples, and the mice were allowed to recover under a heat lamp.

To determine the response range and saturation point for each growth factor, mice were implanted with Elvax pellets containing 0.5, 1.0, 2.0, 5.0, 10, or 20 μg of growth factor. In another series of experiments, pellets contained 10 μg of 17 β-estradiol and 1 mg of progesterone (E/P) in addition to growth factor.

Whole-Mount Preparation of Mammary Gland. Mice were sacrificed 3 days following placement of implants. The entire number 4 inguinal mammary fat pad was removed at the nipple and spread onto a precleaned glass slide. The fat pad was air-dried for 10 min and fixed in acidic ethanol (75% ethanol and 25% acetic acid) for 1 h at room temperature. The tissue was incubated in 70% ethanol for 15 min and ddH_2O for 5 min. Ductal structures were stained in carmine solution [0.2% carmine and 0.5% aluminum potassium sulfate (both from Sigma)] for 12–16 h at room temperature. The stained tissue was dehydrated through graded ethanol, defatted in acetone, and cleared in toluene for 12–16 h. The stained and cleared mammary fat pad was mounted under coverslip with Permount (Fisher) and photographed with a slide duplicator.

Histological Examination. For histological examination of mammary gland ductal structures, fat pads were fixed in 4% paraformaldehyde, stained in hematoxylin or carmine solution, dehydrated through graded ethanol into xylene, and cleared in methyl salicylate (Sigma). Ductal structures identified under a dissecting microscope were excised, blocked in paraffin, sectioned at 4 μm, and stained with H&E using standard procedures.

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